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CALCIUM-DEPENDENT ACTION POTENTIALS IN GIANT SALIVARY GLAND CELLS OF THE LEECH *HAEMENTERIA GHILIANII*: CALCIUM REMOVAL INDUCES DEPENDENCE ON SODIUM

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Summary

1. An electrophysiological study was made of the giant, non-coupled salivary gland cells of the leech *Haementeria ghilianii* (de Filippi, 1849).

2. Resting membrane potential (−40 mV to −80 mV) was primarily dependent on K^+, with a small contribution from a Na^+ conductance and an electrogenic Na^+ pump. Resting Cl^- permeability was low.

3. The cells generated overshooting action potentials (70–110 mV, 100–400 ms) which appeared to be mediated exclusively by Ca^{2+} because they were unaffected by removal of external Na^+ and were blocked by 5 mmol l^{-1} Co^{2+}.

4. Removal of external Ca^{2+} and addition of 1 mmol l^{-1} EGTA produced spontaneous action potentials of reduced amplitude (peaking at about 0 mV) and greatly increased duration [typically tens of seconds but sometimes resulting in sustained depolarizations (plateau potentials) extending up to 30 min or more]. Action potential amplitude was then dependent on external Na^+ concentration, and action potentials were abolished by removal of Na^+. The responses were blocked by 5 mmol l^{-1} Co^{2+}, indicating that they were produced by Na^+ flowing through Ca^{2+} channels.

5. Addition of micromolar concentrations of Ca^{2+} to Ca^{2+}-free saline decreased spike duration and amplitude, suggesting a competition between Na^+ and Ca^{2+}.

6. An electrogenic Na^+ pump was activated by removal of Ca^{2+}, presumably as a result of the influx of Na^+ during spiking; this produced large increases in membrane potential which occurred spontaneously or when Ca^{2+} was reintroduced.

7. In normal saline, spike overshoot and duration were increased when the temperature was lowered by 10°C, whereas in Ca^{2+}-free solution, they were reduced by this change. This suggests that the Ca^{2+} channel may be differentially affected by cooling, depending on the presence or absence of Ca^{2+}.

Introduction

The salivary gland cells of the giant Amazon leech *Haementeria ghilianii* are

Key words: calcium action potential, leech, salivary gland.

extremely large (up to 1·2 mm in diameter) and do not show electrical coupling or dye-coupling (Sawyer *et al.* 1982; Marshall & Lent, 1984; Jones *et al.* 1985). They produce overshooting action potentials that are dependent on calcium, with no measurable contribution by sodium (Marshall & Lent, 1984). For example, spikes are abolished by removal of external calcium but not by removal of sodium or addition of tetrodotoxin; strontium or barium can replace calcium but the spikes are abolished by cobalt, manganese and the calcium antagonist methoxyverapamil.

The action potential seems a likely stimulus for secretion and it is thus important to understand fully its various properties. We therefore decided to extend the studies of Marshall & Lent (1984) on the mechanism of generation of resting and action potentials. It was found that removal of external calcium does not block action potential generation but induces prolonged action potentials which are mediated by sodium. Under normal conditions the spike seems to be mediated solely by calcium. This sodium permeability in the absence of external calcium, which is a feature of certain other calcium channels (e.g. Hess & Tsien, 1984), is shown to be an important consideration in studies of stimulus–secretion coupling in the salivary gland of *Haementeria*.

Materials and methods

Specimens of *Haementeria ghilianii* were obtained from a breeding colony [Biopharm (U.K.) Ltd, Swansea] and maintained in aquaria at 26°C. The aquarium water had the following composition (mmol l^{-1}): NaCl, 0·63; $CaCl_2$, 0·07; $MgCl_2$, 0·07; K_2SO_4, 0·01; Tris-maleate, 2 (pH 6·0) (Sawyer *et al.* 1981).

Preparation

Experiments were performed on the anterior salivary glands which were dissected from the animal together with the posterior glands and a small piece of attached proboscis (see Sawyer *et al.* 1982 for details of the anatomy). The preparation was pinned to the Sylgard base of a Perspex experimental bath (volume 0·25 ml) and immersed in a continuous flow of physiological saline (see below). Solutions were fed into the bath by gravity and removed by aspiration, with a flow rate of about 10 bath volumes min^{-1}. Solutions could be changed rapidly without affecting recording conditions (Holder & Sattelle, 1972). A Ag–AgCl reference electrode was placed in a solution of 3 mol l^{-1} KCl which was connected to the bath *via* a KCl–agar bridge (2 % agar in 3 mol l^{-1} KCl) placed near the outflow.

Electrophysiology

Conventional intracellular recording and stimulating techniques were used. Individual cells were impaled with a glass microelectrode containing 3 mol l^{-1} KCl which was connected to a Digitimer NL 102 amplifier. For current injection, a

second KCl-filled electrode, connected to a similar amplifier, was inserted into the cell and the current was passed and monitored by the amplifier.

Initially it was sometimes difficult to insert a second electrode in spite of the large size of the cells. This problem was also noted by Marshall & Lent (1984) and may result from the extensive infoldings of the cell membrane. A microelectrode beveller was therefore constructed according to the method described by Kripke & Ogden (1974). A 0·3 μm lapping film (Scotch 3M) was used instead of 0·05 μm micropolish, and an acoustic monitoring system was used for controlling the bevelling process (Kaila & Voipio, 1985). Electrode resistances of 60–80 MΩ, measured in saline, fell to 10–20 MΩ after bevelling. This process made microelectrode penetration easier and improved the stability of recordings.

Signals were monitored on a digital storage oscilloscope (Farnell DTS 12) and a pen recorder (Brush 2200S). Most of the records were stored on tape (Thorn EMI 3000 FM tape recorder).

Composition of solutions

The various solutions used are shown in Table 1.

Solutions 1, 2, 3 (except *N*-methyl-D-glucamine; see solution 4) and 5 were adjusted to pH 7·4 by adding about 10 mmol l^{-1} NaOH, bringing the final Na^+ concentration to about 125 mmol l^{-1}. EGTA produced a pH change which was corrected by addition of small amounts of Tris. Solution 4 was adjusted to pH 7·4 with 1 mol l^{-1} HCl. The level of free Ca^{2+} in the Ca^{2+} concentration-buffered salines was 10^{-4} mol l^{-1} for those solutions containing NTA, and 2×10^{-5} mol l^{-1} for the solution containing HEDTA (see Tsien & Rink, 1980; Fukushima & Hagiwara, 1985).

Ouabain (Strophanthin G, 2×10^{-5} mol l^{-1}) was employed as a specific blocker of the Na^+/K^+ exchange pump. Cobalt (II) ions (5 mmol l^{-1} $CoCl_2$) were used to block Ca^{2+} channels. All chemicals were obtained from Sigma Chemical Co. except $CoCl_2$ (BDH Chemicals, Ltd) and $NaCH_3SO_4$ (Hopkin & Williams).

Experiments were performed at room temperature (20–23 °C). For one set of experiments the temperature was rapidly lowered by about 10 °C by switching the incoming flow of saline first through an appropriate length of tubing immersed in ice. A copper–constantan thermocouple was placed next to the preparation in the bath to monitor temperature.

Results

Passive properties

Membrane potential and input resistance

Resting membrane potential varied quite widely from cell to cell, ranging from −40 mV to −80 mV. The lower values did not usually appear to be associated with cell damage and could be sustained for several hours, indicating a genuine wide difference in membrane potential between cells. It should be noted that the cells do not form a homogeneous population but vary widely in size (400–1200 μm in

Table 1. *Composition of solutions (mmol l^{-1})*

	NaCl	Na_2SO_4	Sodium gluconate	$NaCH_3SO_4$	LiCl	*N*-methyl-D-glucamine	KCl	K_2SO_4	Potassium gluconate	$CaCl_2$	$CaSO_4$	Calcium gluconate	Sucrose	EGTA	HEDTA	NTA	Hepes	Tris maleate
1. Normal saline	115						4			1·8								10
2. Ca^{2+}-free solutions	115						4							1				10
						125	4							1				10
	119													1				10
3. Ca^{2+} concentration-buffered salines	115						4			5						10	10	
						125	4			5						10	10	
	115						4			1·8					2		10	
4. Salines with different Na^+ concentrations						125	4			1·8								10
						125	4							1				10
	63					62	4							1				10
	31					94	4							1				10
					125		4							1				10
5. Salines with different Cl^- concentrations		57						2		1·8			76					10
		57						2			10		66					10
			115						4			9						10
				115					4	1·8								10
				115					4			3·6						10

All solutions contained 11 mmol l^{-1} glucose and were buffered at pH 7·4.
HEDTA, *N*-hydroxyethylethylene-diaminetriacetic acid; NTA, nitrolotriacetic acid.

diameter) and histochemistry (Sawyer *et al.* 1982). In a sample of 100 cells from 40 glands the average membrane potential was −56·1 mV ± 9·7 mV (S.D.). In normal saline there was no spontaneous spike activity or evidence of synaptic input such as the miniature potentials found in salivary cells of the cockroach (House, 1980; Ginsborg & House, 1980) and snail (Kater *et al.* 1978*a*).

Cell input resistance was between 2 and 14 MΩ. This value tended to be smaller in the larger cells but also varied with membrane potential (Fig. 1). Resistance increased with hyperpolarization up to about −80 mV and then began to decrease. Cells with high resting membrane potential thus tended to show mainly inward (anomalous) rectification. This non-linear relationship between current and voltage meant that conductance changes produced by any particular procedure had to be checked for indirect effects of changes in membrane potential. Similar current–voltage relationships were found in each of 12 cells that were studied systematically. Results using current pulses were the same as those using a continuous current which was progressively increased under manual control (see

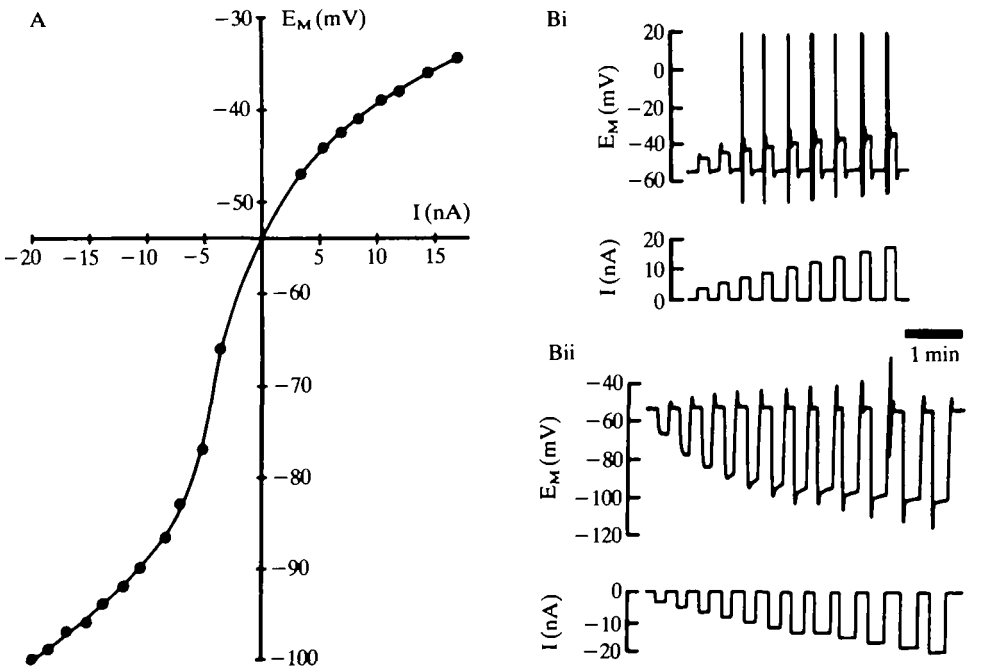

Fig. 1. Typical current–voltage relationships of a gland cell. (A) Plot of steady-state voltage against current; (B) recordings from which the graph is plotted. Upper traces show the potential changes produced by a series of depolarizing (Bi) and hyperpolarizing (Bii) current pulses (lower traces). Note the depolarizing responses, occurring at the end of each hyperpolarizing pulse, which progressively increase in amplitude until a spike (clipped) is produced. With increasing amplitude of hyperpolarizing pulse the membrane potential shows an increasing sag. Depolarizing pulses show delayed rectification.

Marmor, 1975). With larger hyperpolarizing current pulses the potential reached a peak and then rapidly declined to a steady level, indicating a time-dependent inward rectification which increased with strength of pulse (Fig. 1Bii; see Stuenkel, 1985; Bostock & Grafe, 1985). Similar delayed rectification was apparent for subthreshold depolarizing pulses. When a depolarizing or hyperpolarizing pulse was terminated there was a brief ('rebound') hyperpolarization or depolarization, respectively. The transient depolarization increased with the size of the hyperpolarizing pulse until threshold was reached and an action potential was elicited (Fig. 1).

No calculations of specific membrane resistance were made because the membrane is deeply and extensively infolded (Walz *et al.* 1988) so that membrane surface area cannot easily be estimated from cell diameter. In addition, there is the presence of the ductule to consider; this is a single, long process of the cell which leaves the gland and enters the proboscis (Sawyer *et al.* 1982).

Simultaneous recordings from pairs of cells confirmed the results of previous studies that there is no intercellular coupling (Marshall & Lent, 1984; Jones *et al.* 1985). This property is unusual because the cells of almost all exocrine and endocrine glands of vertebrates and invertebrates are coupled to their neighbours (Petersen, 1976, 1980; see Kater, 1977, for an exception). It seemed possible (if unlikely) that the presence of coupling was being masked by effects of pH: low intracellular pH is known to uncouple cells (Bennett *et al.* 1978). Intracellular pH was therefore increased by addition of 20 mmol l^{-1} trimethylamine to the saline (see Thomas, 1984), although no pH measurements were made. [NaCl] was reduced by 20 mmol l^{-1} in compensation, and external pH was maintained at its normal level. However, applied current pulses still failed to pass from cell to cell.

Ionic basis of resting membrane potential

The ionic basis of the resting potential was investigated by varying the concentration of external ions. In agreement with Marshall & Lent (1984), at high external K^+ concentrations the values for resting potential conformed closely to the slope predicted by the Nernst equation for a potassium equilibrium potential. At low K^+ concentrations, however, the membrane potential did not simply deviate from predicted values but showed a depolarization of 1–5 mV in 1 mmol l^{-1} K^+ and 3–10 mV in 0 mmol l^{-1} K^+ (Fig. 2A). This could be attributed to a block of an electrogenic sodium pump (see below).

Na^+ removal produced a hyperpolarization ranging from 5 to 35 mV (mean $15 \pm 6{\cdot}5$ mV, $N = 18$). The maximum level was reached in about 3 min and was associated with an increase in membrane resistance (Fig. 2B); recovery occurred quickly on reintroduction of Na^+. After a few minutes in Na^+-free solution, however, the membrane potential usually recovered slowly, and replacement of Na^+ produced a small transient depolarization. Marshall & Lent (1984) reported no change in membrane potential or input resistance on Na^+ removal but they used a different substitute (arginine). They did not investigate the effects of Ca^{2+}

or Cl^- on membrane potential, and these ions were therefore studied here in some detail.

Removal of Ca^{2+} and addition of 1 mmol l^{-1} EGTA produced a depolarization of 15 mV (±5 mV, $N = 16$) and a reduction in membrane resistance to 54% (±23%, $N = 9$) within about 1 min. Excitability was increased and spontaneous action potentials were usually produced, making the measurements of potential and resistance difficult. Fig. 2C shows an example where spontaneous firing did not occur, and resistance measurements were made with pulses that were too small in amplitude to elicit rebound spikes. The effects of prolonged Ca^{2+} removal on resting membrane potential are described in the next section.

Effects of low-chloride (3·6 mmol l^{-1}) or chloride-free solutions were examined using three different chloride substitutes. Sulphate-based saline produced a

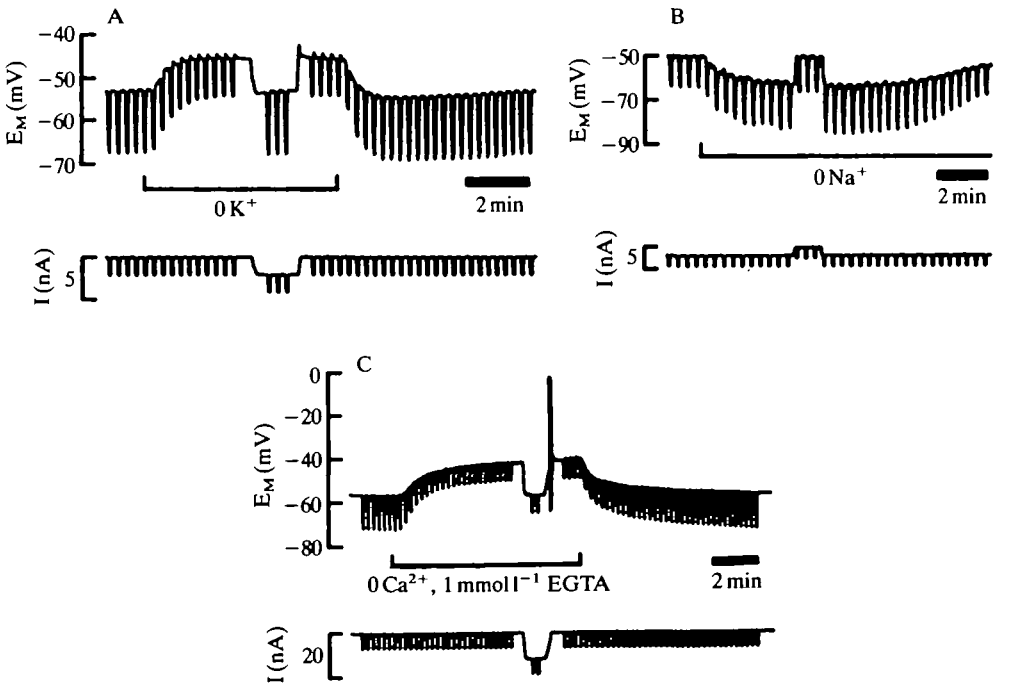

Fig. 2. Effect of removal of external cations on resting membrane potential. Constant-current hyperpolarizing pulses (lower trace) were applied to evaluate membrane resistance. (A) Zero-K^+ evokes a depolarization, indicating a block of an electrogenic sodium pump. The fall in membrane resistance appears to be due mainly to the depolarization since there is no measurable change in resistance when the membrane potential is reset to its original level by applied current. (B) Removal of Na^+ produces a transient hyperpolarization and an increase in membrane resistance; a small increase in resistance is apparent when the membrane potential is reset to its initial level. (C) Ca^{2+} removal produces a depolarization and reduction in membrane resistance. The latter effect is still evident when the membrane potential is briefly returned to its original level by applied current. A short burst of spikes occurs at the termination of the current.

reversible hyperpolarization of 15 mV (±5·5 mV, $N = 20$) which was associated with an increase in membrane resistance (Fig. 3), except in one cell with a high resting membrane potential (−73 mV) where a decrease in resistance occurred. In every case, however, the resistance change was dependent on membrane potential rather than Cl^- removal *per se* (Fig. 3B). Recovery on replacement of Cl^- was faster than the response to its removal, and in some cases there was a small, transient depolarization. Similar results were obtained with a modified sulphate-based saline in which Ca^{2+} and K^+ concentrations were increased by 8·2 mmol l^{-1} and 0·5 mmol l^{-1}, respectively, to allow for their reduced activities produced by sulphate (Hodgkin & Horowicz, 1959; Mullins & Noda, 1963).

With methylsulphate as a Cl^- substitute there was a rapid hyperpolarization of

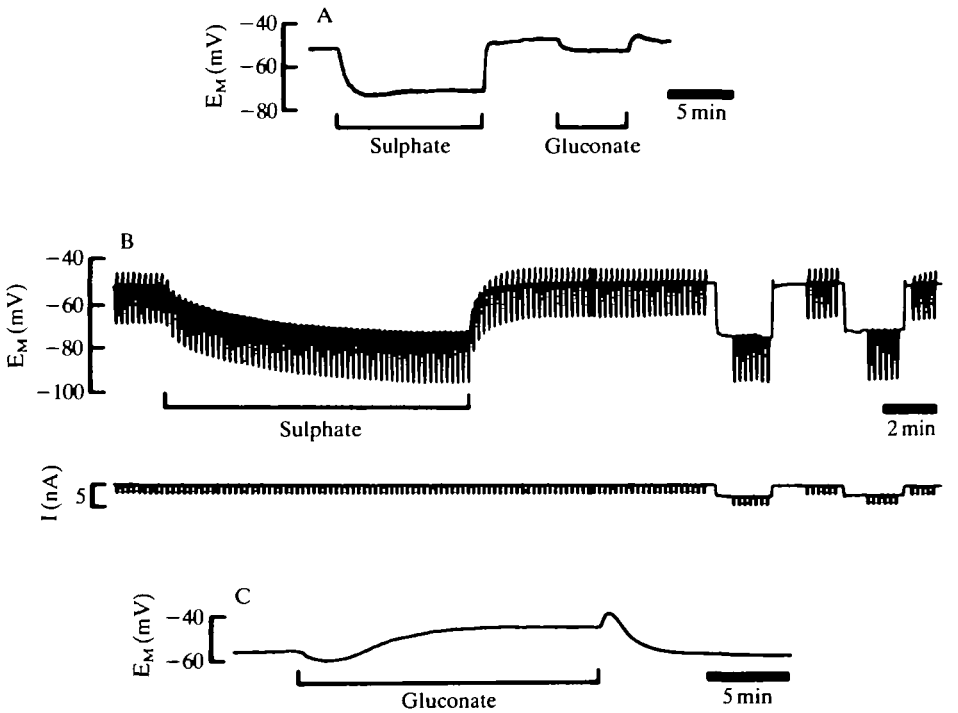

Fig. 3. Effect of removal of external Cl^- on resting membrane potential. (A) Substitution of Cl^- by sulphate or gluconate produces a hyperpolarization. (B) Constant-current hyperpolarizing pulses (lower trace) demonstrate an increase in membrane resistance during the hyperpolarizing response to sulphate. Following recovery, the membrane potential was shifted by applied current to the level attained in sulphate-based solution. The increase in resistance indicates that the changes during Cl^- substitution were the result of change in membrane potential rather than Cl^- removal *per se*. Note that each hyperpolarizing pulse is followed by a small (rebound) depolarization which disappears as the membrane hyperpolarizes. These local responses make it difficult to evaluate the resistance changes by casual inspection. (C) In some cells the hyperpolarizing response to gluconate was transient, and followed by a depolarization. In no case was there an immediate transient depolarization indicative of high Cl^- permeability.

4·8 mV (±2·9 mV, $N = 6$). Gluconate also produced a small hyperpolarization (4·2 ± 1·4 mV, $N = 19$) although in 12 cells this was transient and was followed by a depolarization of 8·6 ± 4·2 mV (Fig. 3). Membrane resistance increased during the hyperpolarizing phase and decreased during depolarization, but again the effects were attributable solely to changes in membrane potential: similar changes were obtained in normal saline by shifting the membrane potential by the same amount with applied current, and when the membrane potential in Cl^--free saline was returned to its initial level there was no measurable change in resistance.

In no case were the responses indicative of a high Cl^- permeability, where one would expect an immediate depolarization on removal of chloride (because of the change in Cl^- equilibrium potential) followed by a hyperpolarization on return to normal Cl^- levels (Hodgkin & Horowicz, 1959; Barber, 1987). The large hyperpolarizations (up to 24 mV) which were found only in sulphate-based saline suggest either that this ion is not totally impermeant or that it has effects of its own. However, the fact that all three substitutes produced a hyperpolarization indicates that this is a true response to Cl^- removal rather than an artefact produced by the replacement ion. No systematic studies were made of its mechanism, but an increase in K^+ permeability appeared unlikely because pulses of 10 mmol l^{-1} K^+ produced similar depolarizations in normal and sulphate-based saline.

In conclusion, the membrane potential of gland cells appears to be largely generated by K^+, with a small contribution from an electrogenic Na^+ pump and a somewhat variable Na^+ permeability.

Action potential

Gland cells produced action potentials in response to depolarizing current pulses or when hyperpolarizing pulses were terminated (anode-break excitation). The overshoot was up to +28 mV, peak-to-peak amplitude between 70 and 110 mV, and spike duration 100–400 ms. Repolarization occurred in two phases and there was a large after-hyperpolarization (see Fig. 4). Impulses produced by a hyperpolarizing or depolarizing pulse were of similar amplitude and configuration, but repetitive activation tended to result in progressively smaller action potentials. Adaptation was rapid, and rarely more than three or four spikes were produced by maintained depolarization.

Marshall & Lent (1984) concluded that the action potential is dependent on Ca^{2+}. We have repeated most of their experiments and can confirm their conclusion. For example, removal of external Na^+ was without effect on spike amplitude, the overshoot was increased by raised Ca^{2+} concentration, Co^{2+} blocked the action potential, and Ba^{2+} and Sr^{2+} could substitute for Ca^{2+}.

Effect of Na^+ removal on normal action potentials

Fig. 4 compares an action potential elicited in normal saline with one recorded after 5 min in saline containing *N*-methyl-D-glucamine in place of Na^+ (when the hyperpolarizing response to Na^+ removal had reached a steady level). Typically there was no effect of Na^+ removal on the rate of rise or amplitude of the action

potential (Fig. 4), although sometimes the amplitude increased slightly. Increasing the concentration of external Na^+ tended to reduce spike amplitude. This indicates a lack of any direct involvement of Na^+ in the generation of the action potential. In four out of nine cells, however, Na^+ removal reduced spike duration by shortening the plateau phase (Fig. 4). Measurements were made for at least 30 min in Na^+-free saline to ensure removal of extracellular Na^+ from the gland, although evidence is presented below that this occurs very rapidly.

Effect of Ca^{2+} removal on normal action potentials

Ca^{2+}-free saline was found not to abolish the action potential but to depolarize the cell and increase its excitability, resulting in action potentials of reduced amplitude (peaking at about 0 mV) and greatly increased duration. Examples of typical responses to Ca^{2+} removal are shown in Fig. 5. In general, spikes progressively lengthened, lasting up to several minutes (Fig. 5A), but in some cases they became much longer (15 min or more) or showed no repolarization until Ca^{2+} was replaced or a large inward current was injected (Fig. 5B,C). The depolarization induced by Ca^{2+}-free solution was associated with a decrease in membrane resistance which became especially pronounced during the peak of the action potential. The depolarizing phase of the spike showed a gradual reduction

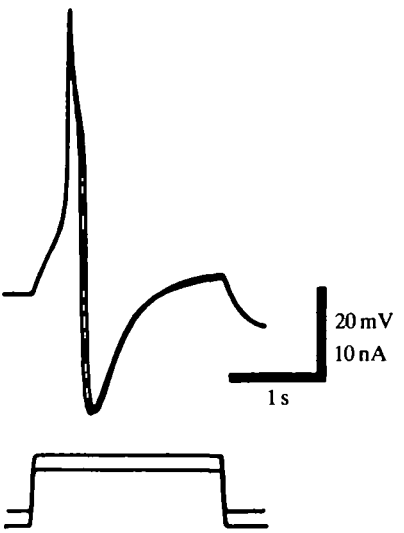

Fig. 4. Effect of removal of external Na^+ on gland cell action potentials. Two superimposed spikes are shown, one elicited in normal saline and the other 5 min after substitution of Na^+ by *N*-methyl-D-glucamine. Typically there is no effect on rising phase or peak depolarization, though in this case the duration was reduced. Stimulating current was the same in both cases (lower trace). The recording in Na^+-free saline was taken when the hyperpolarizing response to Na^+ removal had reached its maximal level; measurements up to 40 min later showed no further effect. Applied current was used to offset the hyperpolarization (6 mV) so that each spike was elicited from the same membrane potential (−48 mV).

associated with a progressive increase in membrane resistance until the spike was suddenly terminated (see first few spikes of Fig. 5C). It was usually possible to predict which action potentials would be greatly prolonged because, after an initial small repolarization (as if about to terminate the spike), the membrane depolarized again and input resistance remained low or decreased further (see longest

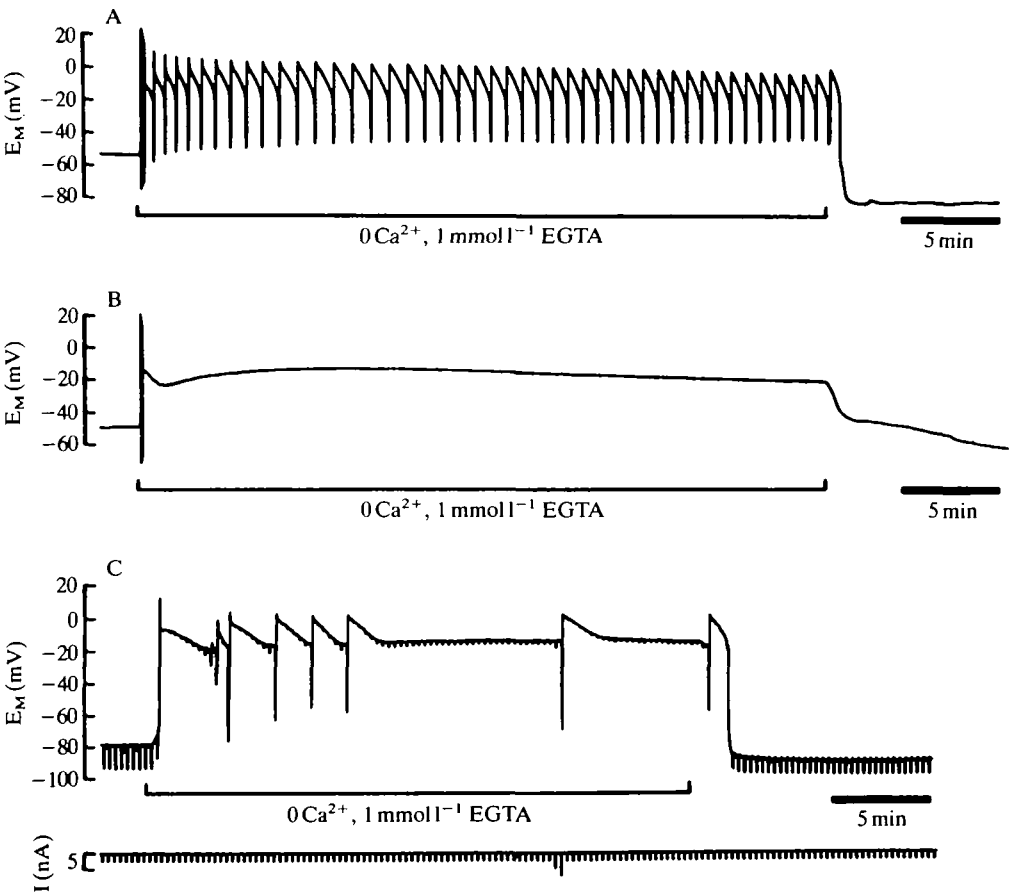

Fig. 5. Effect of removal of external Ca^{2+} on gland cell action potentials. (A,B) Simultaneous recordings from two cells illustrating the two extreme types of response. (A) Typically the cell starts to fire spontaneously and the action potential becomes smaller and much more prolonged. (B) A short burst of action potentials is followed by a plateau potential which lasts for the duration of Ca^{2+} removal. (C) Both types of activity are apparent in this cell (to which constant-current pulses were applied). Each of the first few action potentials is characterized by a long, high-conductance depolarizing phase; there is a slow repolarization and increase in resistance until one of the current pulses terminates the spike shortly before it would otherwise have ended. The longest depolarizing response, however, starts to repolarize and its resistance increases, but it then depolarizes again and the resistance falls. This is typical of a greatly prolonged response which was terminated in this case by the second of the two larger current pulses.

response in Fig. 5C). These long-duration depolarizations with a maintained plateau will be referred to as plateau potentials (see Yang & Lent, 1983).

Effect of Na^+ and Li^+ on action potentials in Ca^{2+}-free saline

The prolonged action potentials produced by Ca^{2+} removal were reduced in amplitude by about 10 mV when external Na^+ concentration was halved to 63 mmol l^{-1} (Fig. 6); they were reduced by a further 10 mV in 31 mmol l^{-1} Na^+ (not shown) and were rapidly and reversibly abolished by complete removal of Na^+ (Fig. 7). Li^+ could substitute for Na^+, producing action potentials of similar amplitude and duration (Fig. 7).

Effect of Co^{2+} on action potentials in Ca^{2+}-free saline

Action potentials in Ca^{2+}-free saline containing either Na^+ or Li^+ were blocked by addition of 5 mmol l^{-1} $CoCl_2$ (Fig. 8). These results suggest that the action potentials are produced by Na^+ or Li^+ flowing through Ca^{2+} channels.

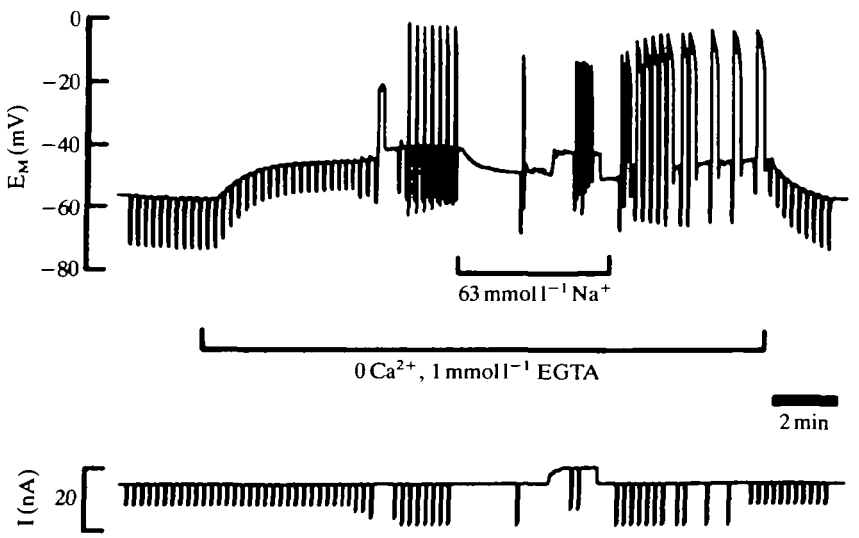

Fig. 6. In Ca^{2+}-free saline the amplitude of gland cell action potentials is dependent on external Na^+. Ca^{2+}-free saline containing 1 mmol l^{-1} EGTA produced the usual depolarization (upper trace) and then the strength of hyperpolarizing current pulses (lower trace) was increased until rebound spikes were elicited (the recording electrode started to come out of the cell and was replaced, with a small loss of membrane potential). Once a plateau had been reached the Na^+ concentration was reduced from 125 to 63 mmol l^{-1} by substitution with *N*-methyl-D-glucamine. A single spike of reduced amplitude was elicited, followed by a burst of spikes. For the latter, current was applied to offset the hyperpolarization caused by reduced Na^+. Gradual recovery of the spike amplitude occurred on reintroduction of saline with a normal Na^+ level, the spikes now being prolonged.

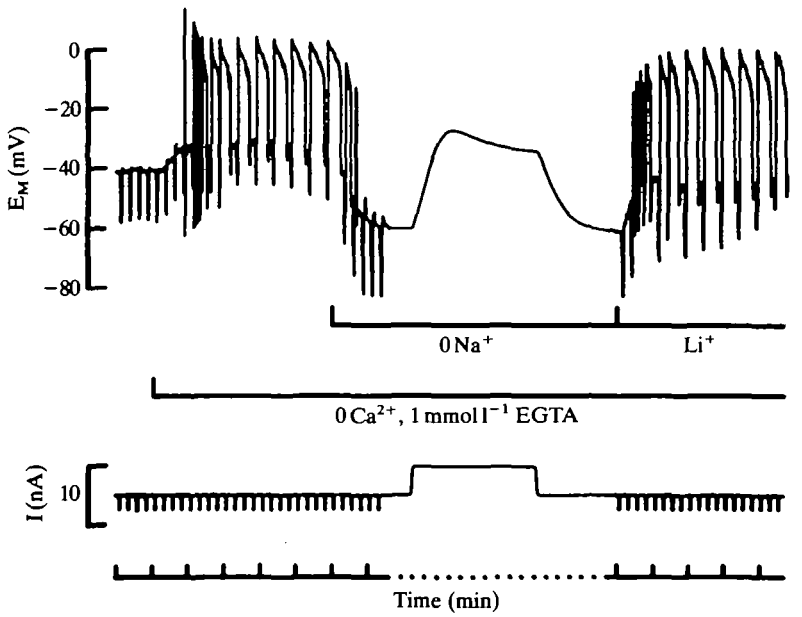

Fig. 7. Action potentials in Ca^{2+}-free saline are blocked by Na^+ removal. Ca^{2+}-free saline (with 1 mmol l^{-1} EGTA) produced a depolarization and characteristic, prolonged action potentials in the gland cell recorded on the upper trace. Applied pulses are indicated by the current trace. Removal of external Na^+ produced a hyperpolarization and block of action potentials. During the period indicated by the dots on the time scale (bottom) the chart speed was increased 100-fold to illustrate that a depolarizing pulse did not elicit a spike even though the potential was shifted to a previously suprathreshold level. Introduction of 125 mmol l^{-1} LiCl in Ca^{2+}-free, Na^+-free solution restored spike activity. Time scale: 1-min intervals are marked.

Effect of micromolar concentrations of Ca^{2+} on action potentials

There are now known to be several examples of normally specific Ca^{2+} channels which become non-selectively permeable to monovalent cations in the absence of Ca^{2+} (e.g. Hess & Tsien, 1984). In such circumstances it has often been found that addition of trace amounts of Ca^{2+} which are too low in concentration to carry significant inward current may block the movement of other ions through the channel (Almers *et al.* 1984). Some indication of this phenomenon was observed in the gland cells when Ca^{2+}-free saline (which, with 1 mmol l^{-1} EGTA, probably contained less than 10^{-8} mol l^{-1} free Ca^{2+}; Miller & Mörchen, 1978) was replaced by a solution containing buffered Ca^{2+} at a concentration between 2×10^{-5} and 10^{-4} mol l^{-1}. Spike duration quickly became reduced and small changes were seen in spike amplitude: in some cases there was an increase but in others a consistent and reversible decrease (Fig. 9). At the concentration of Ca^{2+} which produced these effects, Ca^{2+} did not appear to contribute significantly to the action potential because regenerative responses were abolished by Na^+ removal (not shown). The precise nature of this reduction in spike amplitude and duration by trace amounts

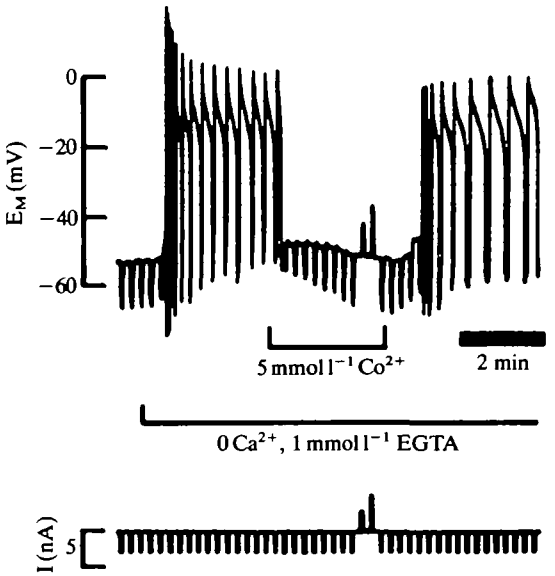

Fig. 8. Na^+ spikes in Ca^{2+}-free saline are blocked by Co^{2+}. Removal of external Ca^{2+} produced a depolarization and the typical Na^+ spikes of reduced amplitude and increased duration. Co^{2+} (5 mmol l^{-1}) produced a small hyperpolarization and increase in membrane resistance. During cobalt treatment neither hyperpolarizing nor depolarizing pulses could elicit action potentials. Recovery occurred on washing out the Co^{2+}. This block of action potentials by Co^{2+} indicates that Na^+ passes through Ca^{2+} channels.

of Ca^{2+} will need to be clarified by measurements of membrane current, but the results are consistent with a block by Ca^{2+} of Na^+ or Li^+ passing through Ca^{2+} channels.

Effect of prolonged exposure to Ca^{2+}-free solution

Ca^{2+} removal produced Na^+ spikes rather than abolition of spike activity. Fig. 10A shows that even after about 1 h in Ca^{2+}-free solution containing 5 mmol l^{-1} EGTA the peak depolarization of the spike showed practically no change. After 1 h there was a steady decline in spike amplitude, probably caused by the associated depolarization rather than removal of Ca^{2+} *per se*. The eventual depolarization and cessation of spiking were not unexpected since prolonged Ca^{2+} depletion is likely to produce deleterious effects, and EGTA may have toxic effects of its own at the concentration used (Miller & Mörchen, 1978).

Fig. 10B shows a recording from a different cell where peak-to-peak spike amplitude *increased* dramatically after immersion for 35 min in Ca^{2+}-free solution. However, for the full 35-min period there was no change in peak depolarization, indicating no tendency for Ca^{2+} removal to abolish action potentials.

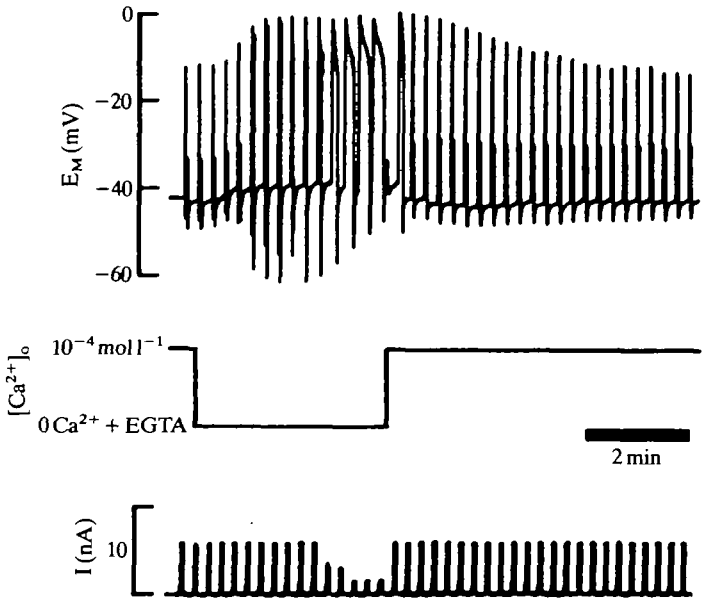

Fig. 9. Action potential amplitude is greater in Ca^{2+}-free solution than in 10^{-4} mol l^{-1} Ca^{2+}. Top trace shows action potentials elicited by depolarizing current pulses in a cell bathed in saline with Ca^{2+} buffered to 10^{-4} mol l^{-1}. In Ca^{2+}-free saline containing 1 mmol l^{-1} EGTA (indicated by middle trace) the spikes increase in amplitude and duration (smaller current pulses were applied because the cell became more excitable). Gradual decline in amplitude occurs on readmission of 10^{-4} mol l^{-1} Ca^{2+}. This indicates that micromolar concentrations of Ca^{2+} do not carry significant current during the action potential but partially block the channel to Na^+.

Activation of an electrogenic Na^+ pump by removal of external Ca^{2+}

The sudden hyperpolarization seen in Fig. 10B is an extreme example, but there was a general tendency for the membrane potential in Ca^{2+}-free saline to increase at some stage beyond the original level found in normal saline: this was in spite of the initial depolarizing response to Ca^{2+} removal. In Fig. 10A, for example, the membrane potential following the second plateau potential is held negative to the initial level for about 5 min. Hyperpolarizations beyond the original membrane potential were also observed after a period of exposure to Ca^{2+}-free solution when normal saline was reintroduced. This is evident in the three recordings in Fig. 5 and for the first (short) period of Ca^{2+} removal in Fig. 10A. In view of the extreme duration of Na^+-dependent action potentials in Ca^{2+}-free saline, and the associated high membrane conductance, it seemed likely that intracellular [Na^+] would rise markedly and that the hyperpolarizations may reflect increased activity of an electrogenic Na^+ pump. In support of this view it was found that treatments that are known to block the pump, such as removal of external K^+ or addition of ouabain, produced a large decrease in potential if applied during the hyperpolarizing phase following Ca^{2+}-free spike activity (Fig. 11). The hyperpolarization and

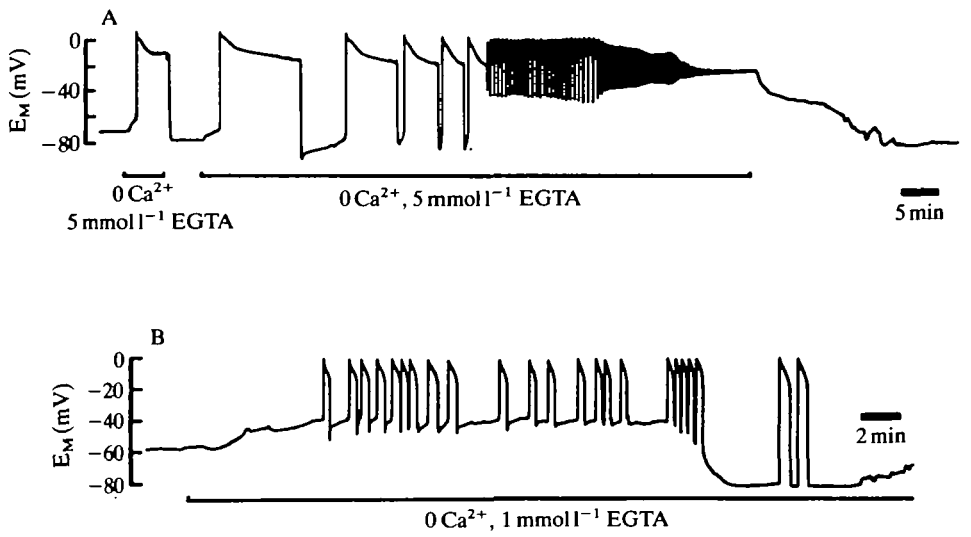

Fig. 10. Effects of longer periods of Ca^{2+} removal on gland cell activity. Recordings are from two cells which showed contrasting responses (the period of Ca^{2+} removal is indicated below each trace). (A) Prolonged action potentials or plateau potentials were produced for about 40 min and then there was an abrupt change to much shorter action potentials that became very small as the membrane progressively depolarized. (B) Prolonged action potentials were again produced but after about 30 min the membrane underwent a large hyperpolarization (off scale) during which two action potentials were produced (in this preparation the gland was attached to the proboscis and the nervous system was intact). Note that for the first 55 min in A and at least 35 min in B there was no change in peak depolarization of the action potential in spite of a large decrease (A) or increase (B) in peak hyperpolarization.

pump activity increased with time spent in Ca^{2+}-free solution (Fig. 11A) and they were especially large if the pump was blocked during the period of firing by removal of K^+ (presumably because this increased intracellular Na^+). Increased activity of the pump was evident for more than 1 h after a 10-min period of Ca^{2+} (and K^+) depletion, and progressively declined as the membrane potential gradually recovered (Fig. 11B).

When Li^+ replaced Na^+ in these experiments, spike activity was not followed by a hyperpolarization or increase in Na^+ pump activity. Since Li^+ is not a substrate for the Na^+ pump (Thomas, 1969) this provides further evidence that the hyperpolarization which follows Na^+ spikes in Ca^{2+}-free saline is indeed generated by a Na^+ pump.

Differential effect of temperature on Ca^{2+} and Na^+ spikes

Calcium channels are known to be very sensitive to temperature (Byerly *et al.* 1984; Narahashi *et al.* 1987). However, little work seems to have been done on the effects of temperature on Ca^{2+} spikes and those produced by non-specific current when Ca^{2+} is removed. We were particularly interested to see whether low

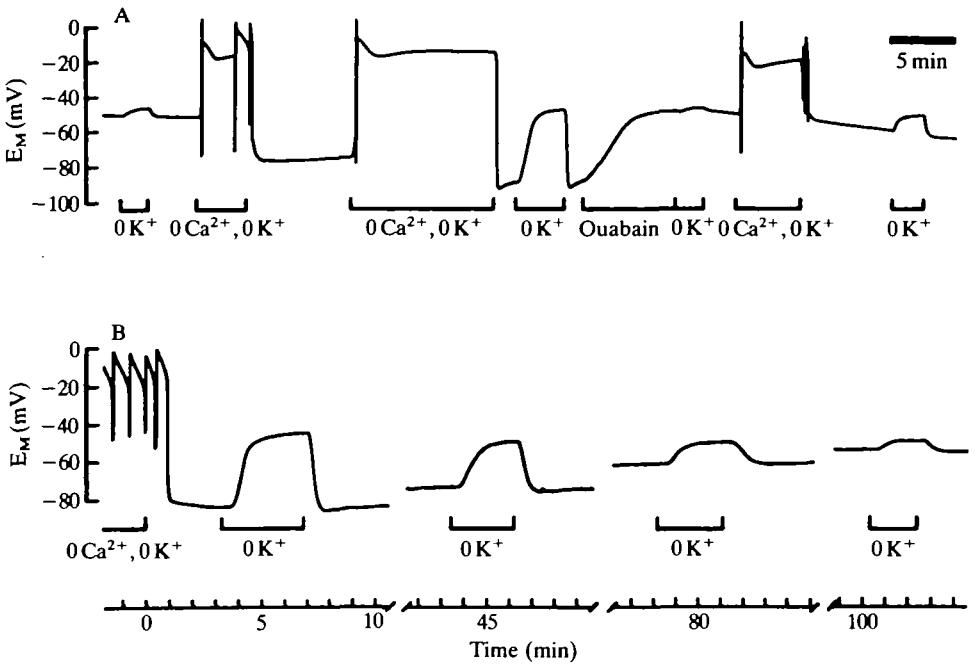

Fig. 11. (A) Na^+ pump activation by removal of external Ca^{2+}. The presence of an electrogenic Na^+ pump is indicated by the small depolarization produced by K^+ removal. This is followed by two periods of exposure to zero-Ca^{2+}, zero-K^+ saline which elicit plateau potentials (K^+ was removed to block the pump and increase the effect of Ca^{2+} removal). Note the increased membrane potential on return to normal saline (especially after the second period) and the slow recovery. During the early recovery period K^+ removal produces a large depolarization indicative of increased pump activity. Subsequent application of 2×10^{-5} mol l^{-1} ouabain produces a similar depolarization and greatly reduces the response to zero-K^+ saline, showing that the pump is substantially blocked. Zero-Ca^{2+}, zero-K^+ saline again produces impulses and a plateau potential but these are not now followed by a hyperpolarization and slow recovery on return to normal saline: there is a slow hyperpolarization as the pump recovers from ouabain. The final application of K^+-free saline shows that pump activity is returning. (B) Change in Na^+ pump activity and recovery of membrane potential following a 10-min period of spiking in zero-Ca^{2+}, zero-K^+ saline (last few spikes are shown). Time zero indicates reintroduction of normal saline. The depolarizing response to K^+ removal, which indicates pump activity, becomes progressively smaller as the membrane potential recovers over a period of about 100 min (full recovery of membrane potential and pump activity occurred at this time).

temperature would reduce the variability of spike duration in Ca^{2+}-free saline by producing predominantly plateau potentials.

The effects of rapid cooling by 10°C are shown in Fig. 12. In normal saline there was a depolarization of 13·5 mV (±5 mV, $N = 19$) and an increase in membrane resistance (despite the depolarization) of 180% (±27%, $N = 16$). Action potentials showed an increase in overshoot of 3–8 mV and a decrease in undershoot of

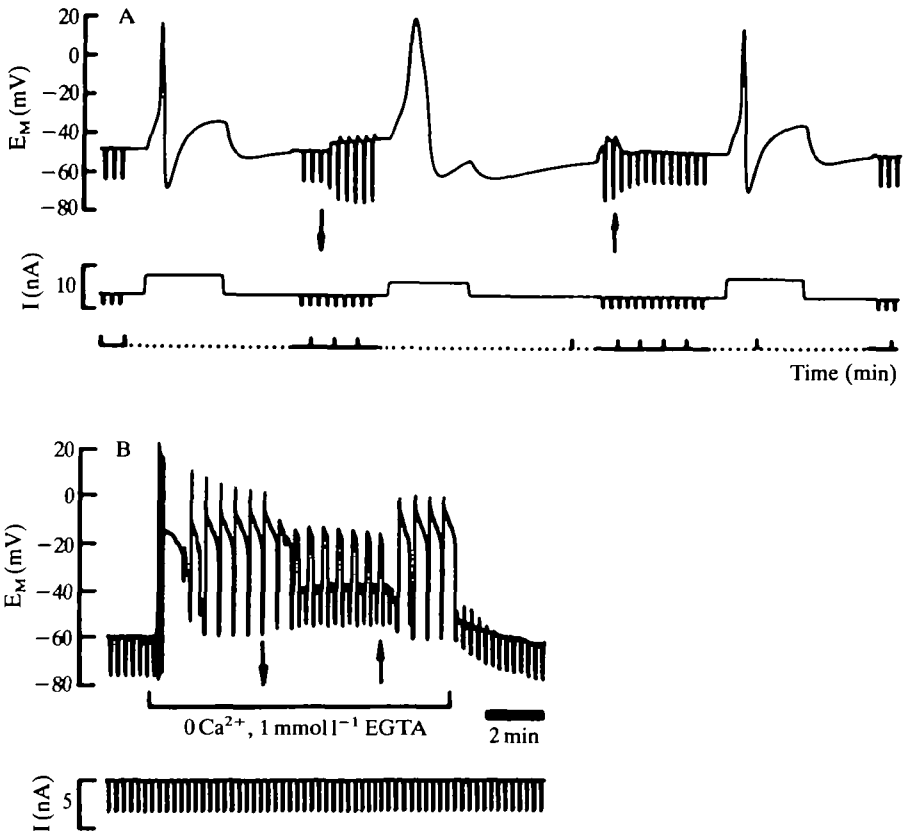

Fig. 12. Differential effects of temperature on Ca^{2+} spikes (normal saline) and Na^+ spikes (Ca^{2+}-free saline). During periods between the arrows the temperature was reduced from 20°C to 10°C. Constant-current hyperpolarizing pulses were applied to monitor membrane resistance. (A) Lowering the temperature of normal saline increased the overshoot, decreased the undershoot, and increased spike duration (spikes produced by depolarizing pulses). During the period indicated by the dots on the time scale the chart speed was increased 100-fold to illustrate spike configuration. (B) Cooling cells in Ca^{2+}-free saline, however, reduced spike amplitude and duration. Note that in this case spikes were terminated prematurely by the current pulses, but cooling did tend to shorten rather than lengthen Na^+ spikes.

4–9 mV; depolarizing and repolarizing phases were slowed and spike duration was increased 3- to 4-fold. In Ca^{2+}-free saline there was also a depolarization (6–9 mV) and increase in resistance on cooling, though spontaneous impulse activity usually made measurements difficult. Peak depolarization of the spike was now reduced in amplitude, however, by 10–15 mV and spike duration was unaffected or reduced (Fig. 12B). This reduction in spike amplitude was much too fast to be caused by a slowing of the sodium pump. The results suggest that the Ca^{2+} channel may be differentially affected by temperature depending on the presence or absence of Ca^{2+}.

Discussion

The purpose of the present experiments is to provide an electrophysiological basis for studies on excitation–secretion coupling in the salivary gland of *Haementeria*, a preparation whose special advantages for this type of work have been well documented (Marshall & Lent, 1984; Jones *et al.* 1985; Sawyer, 1986). Our results confirm the conclusion of Marshall & Lent (1984) that the salivary cell action potential is dependent on Ca^{2+} [most of the criteria suggested by Hagiwara & Byerly (1981) to identify Ca^{2+}-dependent action potentials have been satisfied]. Action potentials were found to persist for an hour or more in zero-Ca^{2+} solution containing 5 mmol l^{-1} EGTA although one of the tests of Ca^{2+} dependence proposed by Hagiwara & Byerly (1981) is a block of the action potential by removal of external Ca^{2+}. There are several examples, however, where this does not occur because the action potential changes to a dependence on Na^+ when Ca^{2+} is removed (Prosser *et al.* 1977; Miller & Mörchen, 1978; Minota & Koketsu, 1983; Yang & Lent, 1983; Yoshida, 1983; Jmari *et al.* 1987). The Na^+ current passes through Ca^{2+} channels which become permeable to monovalent cations in the absence of Ca^{2+} (Hess & Tsien, 1984; Fukushima & Hagiwara, 1985; Lansman *et al.* 1986; Matsuda, 1986; McCleskey *et al.* 1986; Tsien *et al.* 1987). The criterion of Hagiwara & Byerly (1981) that removal of Ca^{2+} should block a Ca^{2+}-dependent action potential is, of course, satisfied if the test is made in the absence of Na^+. It is noteworthy that, in the examples given above, the action potential produced by Ca^{2+} removal tends to be smaller, much longer lasting and more variable in duration than normal, but maintains a fairly rapid repolarizing phase (as in *Haementeria*).

Although the ability to pass non-specific current may be a general property of Ca^{2+} channels (Almers & McCleskey, 1984), there are several examples of Ca^{2+}-dependent action potentials that do become blocked by Ca^{2+} removal (Patlak, 1976; Weisblat *et al.* 1976; Fukuda *et al.* 1977; Mizunami *et al.* 1987) or become greatly attenuated (Hadley *et al.* 1980; Goldring *et al.* 1983). Thus there remained the possibility in the present experiments that failure of the action potential to disappear in Ca^{2+}-free solution was due to incomplete washout of Ca^{2+} from the gland. This is most unlikely, however, because a depolarization and increase in membrane conductance occurred within a minute or two of Ca^{2+} removal, and the peak depolarization of the action potential rapidly declined to a level which remained steady for about 1 h, even during large changes in spike undershoot. Most importantly, Na^+ removal barely affected the normal spike but produced a rapid, reversible block of impulse activity in Ca^{2+}-free saline. Finally, activation of the Na^+ pump by impulse activity, and the rapid effect on spike configuration of changing from Ca^{2+}-free to low-Ca^{2+} solution are also explained in terms of effective exchange of Ca^{2+} in the gland. In Ca^{2+}-free solution the salivary cell action potentials are maintained by Na^+ or Li^+ which appear to pass through Ca^{2+} channels because the spikes are blocked by 5 mmol l^{-1} Co^{2+}.

It is worth reiterating that studies on the salivary cells of *Haementeria* are free from the problems of electrical coupling which may seriously complicate the

interpretation of results in other glands. For example, Hadley *et al.* (1980) could not perform reliable quantitative measurements of action potential characteristics in salivary cells of *Planorbis* because spike amplitude and configuration varied with the number of neighbouring cells which were firing (functional coupling tends to become reduced when cells fire synchronously; see Getting, 1974). In many circumstances it is difficult to determine whether recorded electrical activity comes from the impaled cell or spreads from coupled neighbours; this problem is particularly serious if different cell types are present, when even qualitative changes may become difficult to interpret. Ion substitution experiments may be complicated by effects on coupling resistance in addition to membrane resistance (removal of external Ca^{2+} or Cl^-, for example, may produce uncoupling, with a resultant increase in input resistance; Asada & Bennett, 1971). None of these problems occurs in *Haementeria*, and the absence of innervation in the gland (W. A. Wuttke, R. T. Sawyer & M. S. Berry, in preparation) eliminates problems of indirect actions on presynaptic elements (see Ascher *et al.* 1976).

Electrical excitability is not a feature of mammalian exocrine glands (Petersen, 1980) but is found in certain endocrine gland cells such as the pancreas (Matthews & Sakamoto, 1975), adenohypophysis (Kidokoro, 1975) and adrenal gland (Brandt *et al.* 1976). Among invertebrates, the salivary glands of insects are inexcitable (Ginsborg & House, 1980; House, 1980) whereas those of molluscs produce action potentials (Kater *et al.* 1978*b*; Goldring *et al.* 1983; Barber, 1983). Molluscan pedal gland cells (which secrete mucus) also produce impulses (Kater, 1977). Electrically excitable secretory cells invariably seem to have a Ca^{2+} component to their spikes, although there may also be a large or small contribution by Na^+. In *Haementeria*, only a Ca^{2+} component is evident.

It is generally accepted that action potentials in gland cells act as a stimulus for secretion by providing an influx of Ca^{2+} which is necessary for exocytosis (Hagiwara & Byerly, 1981). The situation is analogous to Ca^{2+} entry into the presynaptic element of a nerve cell (the similarity is particularly marked in *Haementeria* where the impulse travels along a ductule towards the release site in the proboscis). We were surprised, therefore, to find in our early experiments that Ca^{2+} removal increased the amount of secretory product around the tip of the proboscis (W. A. Wuttke & M. S. Berry, unpublished results), but this accords with our present findings that Ca^{2+} removal actually excites the gland cells. There remains the problem, however, of the mechanism of secretion in the absence of external Ca^{2+}. One possibility is that Na^+ influx results in the release of Ca^{2+} from intracellular stores and this triggers release of secretory products (Lowe *et al.* 1976). The most likely explanation, however, is that Ca^{2+} is not easily washed from the compact, muscular proboscis. Stimulation of the nerve to the proboscis elicited contractions after more than 30 min in Ca^{2+}-free saline containing 5 mmol l^{-1} EGTA, indicating the presence of sufficient Ca^{2+} for release of neurotransmitter. Long periods have also been found necessary to remove Ca^{2+} from ganglia in *Aplysia* (Kehoe, 1969). It must be stressed, however, that although our results may be in agreement with a Ca^{2+}-dependent, impulse-evoked release

of secretion, they do not provide any positive evidence. Experiments are under way on the mechanism of secretion in *Haementeria* but little is currently known.

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